Conformational analysis of the hydrophobic peptide α_{s1}-casein(136–196)[1]

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Abstract

Hydrophobic interactions are important in the self-association of milk proteins, including α_{s1}-casein. The extent to which casein interaction sites are influenced by local secondary structure is not widely known. Both primary amino acid sequence and local secondary structure are shown to affect the self-association of the hydrophobic peptide α_{s1}-casein(136–196). The peptide is aggregated at low concentrations (7 μM and above), as determined by ^{1}H nuclear magnetic resonance (NMR) measurements at pH 6.0 in phosphate buffer. Increase in temperature is shown to induce side chain mobility (melting) as indicated by both ^{1}H NMR and near-UV circular dichroism (CD) measurements. As determined by far-UV CD, there is also a loss in the global amount of extended structure with increasing temperature, while β-turn structures and some aromatic dichroism are conserved at temperatures as high as 70°C. Similar retention of structure occurs at pH 2 and in 6 M guanidine HCl. The observed stability of β-turns and some side chains in α_{s1}-casein(136–196) supports previous assumptions that hydrophobic, proline-based turns are important interaction sites in the self-association of α_{s1}-casein, and possibly in the formation of the calcium transport complexes, the casein micelles. It may be speculated that these areas of the peptide represent a 'molten globule-like', heat stable, core structure for α_{s1}-casein.

Keywords: Casein; Conformation; Cyanogen bromide cleavage; Nuclear magnetic resomance; Circular dichroism; Fourier transform infrared spectroscopy; Peptide conformation; Molten globule

1. Introduction

The caseins are a unusual group of proteins, which are neither globular nor fibrous in nature. Caseins have an open structure and are hydrated despite having a high content of hydrophobic amino acids. These proteins also have areas of high net negative charge. The combination of electrostatic and hydrophobic properties allows the caseins to form large colloidal aggregates, while sequestering inorganic calcium and phosphate for transport to the neonate [1,2]. The high calculated hydrophobicities and the proline contents of the caseins are thought to prevent the formation of globular structures in which the nonpolar groups are completely buried in the hydrophobic interior of the protein [3,4]. Thus a number of the hydrophobic amino acids in caseins are found on

Abbreviations: CD, circular dichroism; FTIR, Fourier transform infrared; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NOESY, nuclear Overhauser enhanced spectroscopy

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the outer molecular surface of casein monomers. These hydrophobic surfaces on the molecule are important in casein-casein interactions and may account for the self-associative properties, which lead to colloid formation. The most abundant milk protein, α_{s1}-casein (B variant), is a single-chain polypeptide of known sequence [3], with 199 amino acid residues and a molecular mass of 23 619. The primary sequence of the α_{s1}-casein B variant is given in Fig. 1. The major calcium binding peptide is located in the N-terminal half of the molecule and has been studied in detail [5,6]. The high degree of hydrophobicity for the C-terminal half of the molecule (residues 100–199) is thought to be responsible for the pronounced self-association of the α_{s1}-casein monomer in aqueous solution [1,2]. The role of aromatic amino acid side chains in stabilizing nonpolar regions of self-associated α_{s1}-casein and α_{s1}-casein peptides was suggested in a previous report [7].

It may be anticipated that secondary and tertiary structures would be important factors in casein interactions. Secondary structural features could influence casein monomer self-association, while tertiary structures may control the formation of casein submicelles, an intermediate step in the formation of the colloidal calcium transport complex [1,2,6]. Three-dimensional structures of proteins have been realized primarily by X-ray crystallography and high-resolution nuclear magnetic resonance (NMR) spectroscopy. However, to date caseins have never been successfully crystallized, and consequently crystallographic structures are not likely to be available. A lack of well-defined and stable casein structures has led to somewhat slow progress in NMR structural work. Advances in the structural studies of milk pro-

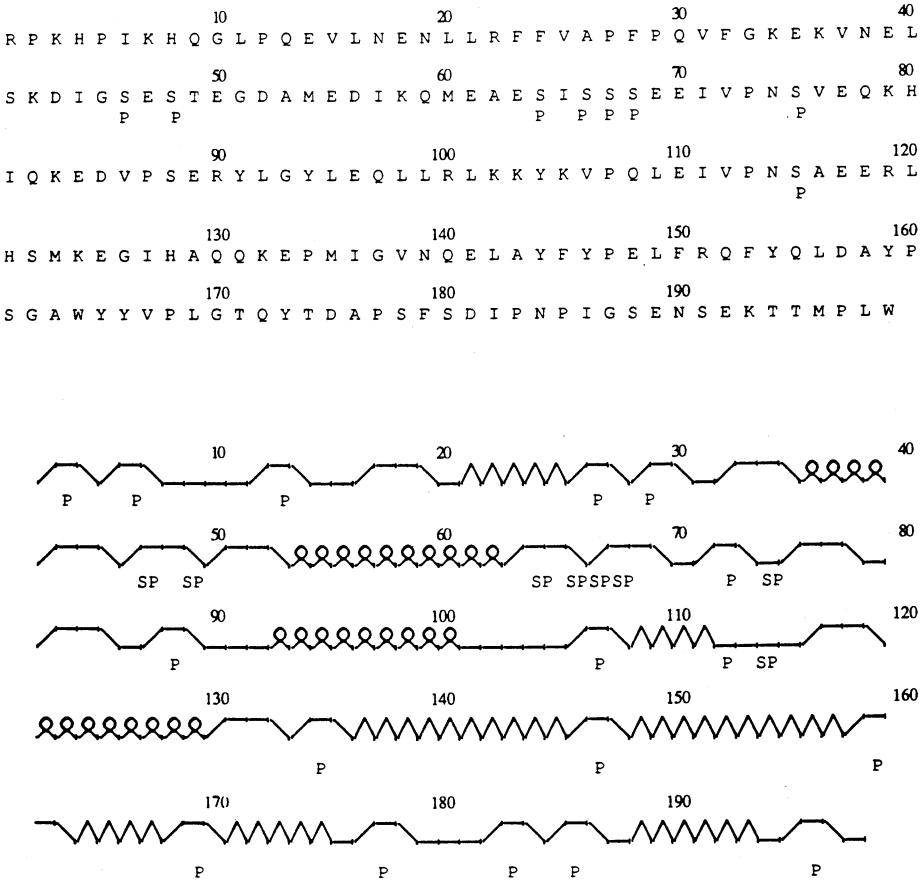

Fig. 1. (A) Sequence of α_{s1}-casein B; P below S denotes serine phosphate [3]. (B) Summary of initial secondary structural assignments made for α_{s1}-casein. P denotes proline; SP denotes serine phosphate; α-helix denoted by scroll (e.g., residues 37–40); β-sheet by sawtooth (e.g., residues 21 to 25); turns by turn-like structures (e.g., residues 16–19).

teins were surveyed in a recent review by Creamer and MacGibbon [8]. Natural abundance NMR studies of casein micelles have proven difficult to interpret due to spectral crowding, although protein regions with substantial conformational mobility can be resolved from rigid core regions [9–12]. A limited number of structural studies have been done on small casein peptides, both synthetic and those obtained from chemical or enzymatic digests, including κ-casein(98–111) [13], κ-casein(130–153) [14], the β-casein phosphopeptides: β-casein(1–25) [15,16] and β-casein(1–42) [17], and the α_{s1}-casein phosphopeptide α_{s1}-casein(59–79) [5]. Most NMR results indicate few populations of stable secondary structures. Random coil and extended structures were predicted for most peptides [13–17], although isolated β-turn and loop structures, as well as some α-helix, were detected for α_{s1}-casein(59–79) [5].

Caseins have been thought to be 'random' structures with only small amounts of secondary structures because the caseins are characterized by a high content of proline residues. Proline is known to disrupt regular structure but excels at forming turns [18]. Previous studies of caseins by Raman [19] and infrared [20] spectroscopies indicate a high degree of turns, as well as more β-sheet than previously expected in casein monomers and polymers. Proline-directed reverse turns in caseins may be of particular significance, as it has been postulated that reverse turns play an important role in initiating and directing protein folding [21]. β-Turns generally occur on the solvent-exposed surface of proteins, and are usually in hydrophilic regions of the polypeptide chain. Caseins, however, tend to self-associate, with the formation of hydrophobic cores in the associated complexes. It has been postulated that proline turns in hydrophobic areas can appear on the surface of casein monomers, which, in turn, may be buried in subsequent polymer formation. Exposed hydrophobic sites on casein monomers help to explain the nature of casein interactions, and may be the structural motif which sets these proteins apart from both globular and fibrous proteins. Using the known characteristics of α_{s1}-casein, an energy-minimized predicted structure generated for the prevalent B variant of α_{s1}-casein [22,23], is given in Fig. 2. The possible conformational states for the individual amino acid residues were determined using sequence-based predictive techniques. The apparent correlation between the percent proline and the percentage of predicted turns was used as rationale for assigning proline residues to turn structures in the model. It should be stressed that the published energy-minimized structure in Fig. 2 is a working model, subject to refinement.

In a previous report [7], the influence of tyrosine and tryptophan side chains on the formation of α_{s1}-casein aggregates was shown through investigation of the hydrophobic peptide α_{s1}-casein(136–196). This peptide was isolated from the parent α_{s1}-casein after chemical cleavage with cyanogen bromide. Aromatic side chains from residues neighboring proline-based turns in the putative molecular model for native α_{s1}-casein (Fig. 2) were shown to be involved in the hydrophobic self-association of α_{s1}-casein(136–196). Tyrosine residues in the vicinity of Pro147 appear to be involved in the initial dimerization of the peptide, while the region surrounding Trp164 appears to be a key factor in the formation of higher-order aggregates at high ionic strengths and peptide concentrations. The possible role of hydrophobic β-turn conformers in initiating peptide folding and directing subsequent association is investigated in this study by ^{1}H NMR, far-UV CD and FTIR spectroscopies.

2. Materials and methods

2.1. Materials

All reagents used were of analytical grade or 'ACS certified' from Sigma (St. Louis, MO). Isolation and purification of both α_{s1}-casein and the cyanogen bromide cleavage peptide α_{s1}-casein(136–196) were presented in some detail in Section 2 of our previous paper [7].

2.2. NMR measurements

A stock sample for NMR spectroscopic analysis was prepared by dissolving a known amount of lyophilized peptide in 0.4 ml of a buffered (10 mM Na_2HPO_4, pH 6.0) 90% H_2O/10% D_2O solution. Samples of 73 μM (0.51 mg/ml), 37 μM (0.26 mg/ml) and 7 μM (0.05 mg/ml) peptide concentration were prepared by dilution, with final concentrations

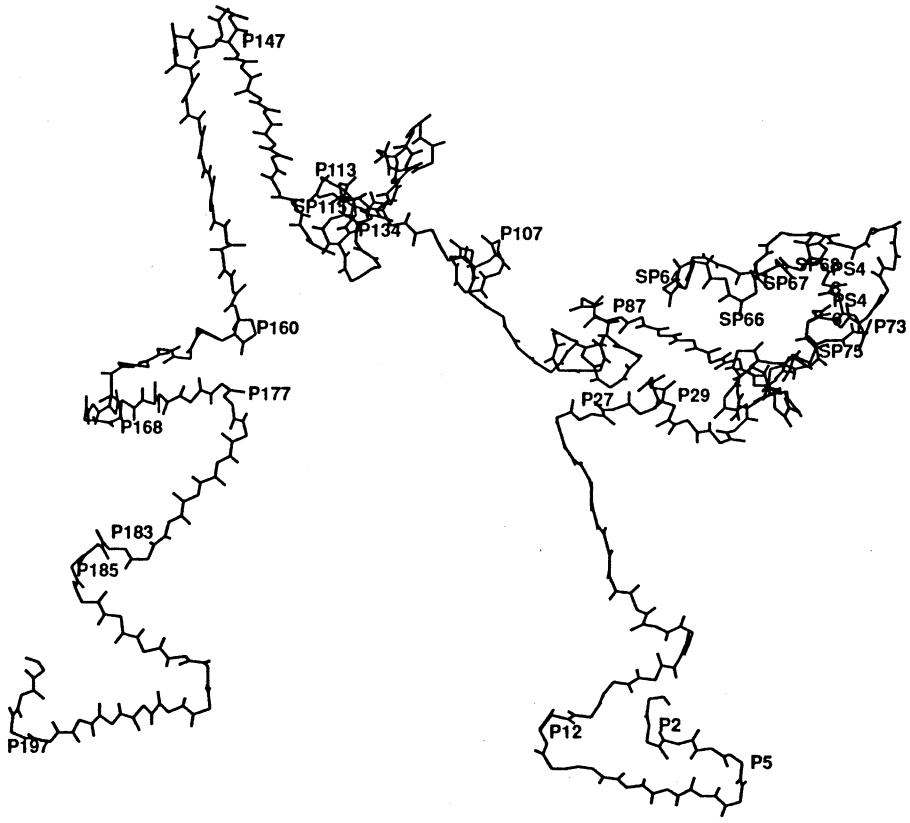

Fig. 2. Energy-minimized putative three-dimensional molecular of α_{s1}-casein. The peptide backbone only is displayed. Proline residues are denoted by P, while phosphoserine residues are denoted by SP [22,23].

determined from analysis of their ultraviolet (UV) absorption spectra using an extinction coefficient of 1.8 ml/mg at 283 nm calculated from amino acid composition. For initial structural studies, a 0.4 ml aqueous sample of 1.5 mM (10.5 mg/ml) peptide was prepared in 10 mM Na_2HPO_4 buffer at pH 6.0. Adjustments in pH were made with NaOD and/or DCl. Spectra for the 37 μM, pH 6.0 sample were obtained for a variable temperature range of 10–70°C. An additional sample of 37 μM peptide was made up in 20% methanol/80% D_2O.

All NMR experiments were performed on a Bruker AMX 600 NMR spectrometer (Billerica, MA) equipped with a 5 mm broadband inverse probe, at a ^1H frequency of 600.1 MHz, respectively, using the XWIN-NMR 1.1.1 software package run on a Silicon Graphics (Mountain View, CA) INDY workstation. Typically, one-dimensional (1D) ^1H spectra were obtained with a total repetition time of 2.5–3.5 s and presaturation of H_2O solvent signal. All 1D ^1H NMR data were Fourier transformed with 0.3–1.0 Hz exponential line broadening. Proton chemical shift values were referenced to external 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Phase-sensitive 2D nuclear Overhauser enhancement spectroscopy (NOESY), total correlation spectroscopy (TOCSY), and double quantum filter correlation spectroscopy (DQF-COSY) experiments were obtained using the TPPI method of data acquisition [24]. Mixing times were 300 ms and 124 ms for the NOESY and TOCSY experiments, respectively. Two-dimensional experiments were typically carried out at 10°C under the following conditions: 2K data points in t_2, 1K data points in t_1 with 24 scans per increment, a total repetition time of 5.2 s, and presaturation of the H_2O signal. Data from 2D experiments were processed with 90° (for TOCSY and DQF-COSY) and 45° (for NOESY) shifted sine bell functions in both dimensions followed by automatic baseline correction in F1 and F2.

2.3. Circular dichroism (CD) measurements

Far-UV CD experiments were carried out with 0.3 mg/ml peptide samples in a 2 mM PIPES (piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)), 4 mM KCl buffer at pH 6.75. Successive measurements in the far UV (190–250 nm) were made at 10°C, 27°C, 50°C and 70°C. Solvents for CD measurements were first filtered through a Millipore 0.22-μm pore filter. Dissolved peptide samples were filtered through centrifuge tubes containing a 0.45-μm pore regenerated cellulose filter. Circular dichroism spectra were recorded on an Aviv model 60DS spectropolarimeter (Aviv Associates, Lakewood, NJ) at 25°C, using cells of appropriate path lengths and a scan time of 40 s/nm. The jacketed cells were attached to a circulating constant temperature bath; the time for equilibration of the sample was calculated to be 30 min for a 30°C change in the bath temperature. Spectra are corrected for solvent contributions and are expressed in units of mean residue ellipticity (far-UV) or molar ellipticity (near-UV) vs. wavelength. Analysis of peptide secondary structure from CD spectra was accomplished using the CONTIN procedure of Provencher and Glöckner [25].

2.4. Infrared (FTIR) spectroscopic measurements

For infrared (FTIR) measurements, 3 mg of α_{s1}-casein(136–196) were dissolved as a 1.5% w/w aqueous solution at pH 6.75 in 25 mM dipotassium PIPES buffer. Spectra were obtained at 25°C using a Nicolet 740 FTIR spectrometer (Madison, WI) equipped with a Nicolet 660 data system. Data collection was carried out after a nitrogen purge of the sample chamber, which consisted of a demountable cell with CaF_2 windows separated by a 6-μm Teflon spacer. Each spectrum consists of 4096 double-sided interferograms, co-added, phase-corrected, apodized with a Happ–Genzel function, and fast-Fourier transformed. Secondary structural features were calculated from the amide I envelope, after assignment of putative peaks by use of second derivative spectra, and fitting of Gaussian peaks to the original spectra. The selected peaks were refitted to the original spectra using an iterative curve-fitting procedure as previously reported [26]. Data are reported as the sum of assigned spectral elements gathered from a 20-protein data base [26].

3. Results and discussion

3.1. Characterization of the α_{s1}-casein(136–196) peptide by ^1H NMR spectroscopy

The α_{s1}-casein(136–196) peptide has a high propensity for self-association, as demonstrated previously by sedimentation and near-UV CD measurements [7]. It would be advantageous for ^1H NMR studies to obtain the peptide in a monomeric form, so as to eliminate ambiguity in the interpretation of NOESY experiments. Previous results [7] from sedimentation equilibrium experiments on α_{s1}-casein(136–196) indicated the peptide to be predominantly dimer (and a small population of oligomere) under the following experimental conditions: 1 mg/ml (0.15 mM) aqueous peptide solution, pH 6.75, $T = 25$°C, low ionic strength (10 mM PIPES buffer, $\mu = 0.0175$ M).

Investigations of this peptide in aqueous solution were carried out under conditions suitable to structural analysis by 2D NMR methods. A one-dimensional ^1H NMR spectrum was obtained for a 1.5 mM peptide solution in 10 mM sodium phosphate buffer at pH 6.0, $T = 10$°C. Proton spectral features were rather broad for a peptide of this size ($M_r = 6995$). Fig. 3 shows the NH/C_αH fingerprint region of the 600 MHz NOESY spectrum (mixing time = 300 ms) for α_{s1}-casein(136–196). The F2 axis (backbone amide proton region) also includes a projection of the 1D ^1H spectrum. There are approximately 25–30 discernible peaks in the NOESY fingerprint region, which is considerably less than expected for a 60-residue peptide. The broad 1D spectral features and the lack of NOE cross-peaks both suggest that the peptide is somewhat more aggregated than expected. While 2D TOCSY and DQF-COSY (data not shown) experiments were useful in assigning the identity of several amino acid spin systems, the lack of sequential NOE connectivities precludes a complete spectral assignment of all peptide residues.

Aromatic amino acid side chains, particularly tyrosine and tryptophan, are major constituents of the hydrophobic cores of aggregated or folded proteins

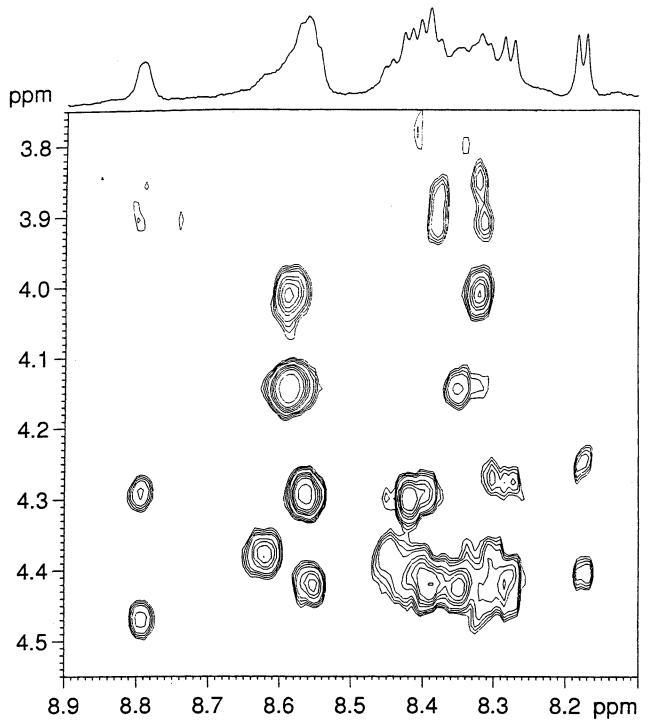

Fig. 3. The NH/C_αH fingerprint region of the 600.1 MHz NOESY spectrum (mixing time = 300 ms) of 1.5 mM α_{s1}(136–196) casein peptide in 90% H_2O/10% D_2O. The spectrum was obtained at 10°C, pH 6.0, in the presence of 10 mM Na_2HPO_4. The upper portion of the figure shows the amide proton region for corresponding one-dimensional proton spectrum of 1.5 mM α_{s1}(136–196) casein obtained under the same conditions as the NOESY spectrum.

and peptides. Tyr and Trp rings can pack against one another, as well as against hydrophobic regions of the polypeptide backbone and/or side chains. In the first part of this study [7], large perturbations in the near-UV (250–320 nm) CD spectra were observed upon formation of high-order aggregates (degree of polymerization ≥ 5) for α_{s1}-casein(136–196), and Trp-164 was indicated as a possible site for these interactions. Fig. 3 shows the 1D ^1H region representing aromatic and side chain amine protons for various solutions of α_{s1}-casein(136–196) in H_2O. In order to improve the spectral characteristics of this peptide, several strategies were pursued. Increasing the pH to 7.0 and/or recording the spectra at 30°C did not improve the appearance (Fig. 4A,B). This is particularly evident from the broad aromatic resonances, recorded for the sample in D_2O (Fig. 4B) which upon closer inspection are also present in Fig. 4A. These spectra indicate that α_{s1}-casein (136–196) is multimeric at mM concentrations. In order to investigate the multimer formation in more detail by NMR, spectra were recorded for dilute solutions in D_2O. The dilution series included the following samples: 80 μM to 7 μM α_{s1}-casein(136–196) in D_2O. Proton spectral features sharpen only marginally upon lowering the concentration from 1.5 mM to 80 or 32 μM (Fig. 4C,D). Even at 32 μM, the breadth of peptide backbone resonances (C_αH) indicates a fairly high degree of polymerization. For some small proteins and linear peptides, the presence of an organic cosolvent can have a considerable effect on the conformation in solution. Methanol was used as a cosolvent in an attempt to disrupt intermolecular hydrophobic interactions for α_{s1}-casein(136–196). The observed spectral features did not sharpen to any extent for a 40

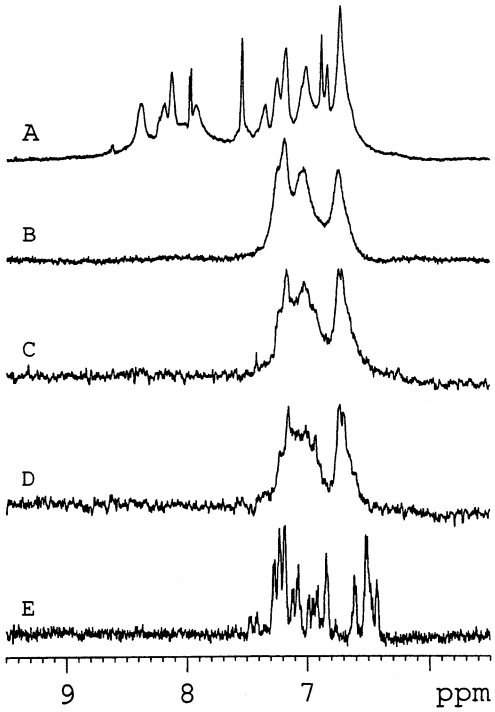

Fig. 4. The 1D ^1H region representing aromatic and exchangeable protons for (A) 1.5 mM α_{s1}-casein(136–196) in H_2O (10 mM sodium phosphate, pH 6.0); (B) 1.5 mM α_{s1}-casein(136–196) in D_2O (same conditions as A); (C) 80 μM α_{s1}-casein(136–196) in D_2O (same conditions as A); (D) 32 μM α_{s1}-casein(136–196) in D_2O (same conditions as A); (E) 40 μM α_{s1}-casein(136–196) in D_2O (pH 11.0). All spectra were recorded at 30°C.

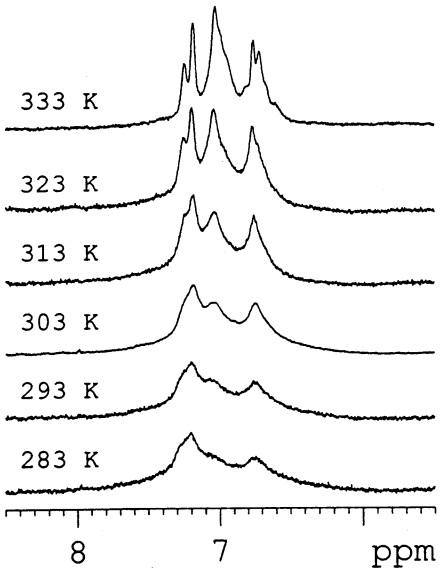

Fig. 5. The 1D ^1H region representing aromatic protons for 1.5 mM α_{s1}-casein(136–196) in D_2O (10 mM sodium phosphate, pH 5.6) as a function of temperature.

μM solution of α_{s1}-casein(136–196) in 20% methanol/80% D_2O (data not shown). As anticipated, recording a sample of 40 μM α_{s1}-casein(136–196) at pH 11.0 produces comparatively sharp peaks indicating that the intermolecular interactions are disrupted (Fig. 4E) at high pH.

3.2. *Effects of temperature on the NMR spectra of the α_{s1}-casein(136–196) peptide*

The self-association of α_{s1}-casein has been shown to be nearly temperature independent between 8°C and 30°C, suggesting that electrostatic as well as hydrophobic forces play a role in self-association of the native protein [1,2]. Because the highly charged phosphopeptide is absent, hydrophobic interactions should dominate the self-association of the α_{s1}-casein(136–196) peptide, thus making intermolecular interactions highly temperature sensitive. Fig. 5 shows the downfield proton region for spectra of 1.5 mM α_{s1}-casein(136–196) in D_2O for a series of temperatures. The pH (5.6) and buffer conditions (10 mM sodium phosphate) are constant for the temperature series. The aromatic resonances (6.5–8.0 ppm) sharpen at elevated temperatures. Resonances in the backbone C_α proton region (not shown) exhibit the same temperature-dependent features.

The sharpening of much of the proton spectrum for α_{s1}-casein(136–196) as a function of temperature is an interesting occurrence. For proteins in general, heating leads to denaturation and peak broadening, but 'side chain melting' was the basis for the introduction of the term 'molten globule' [27]. Swelling of the polypeptide chain with increased temperature could lead to increased side chain mobility. It had been noted that some sharpening of whole casein ($M_r > 250\,000$) NMR bands occurred on heating [10,11]. These workers suggested that this phenomenon might be the result of dissociation of protein monomers from the aggregate, a result of heat treatment. An alternative view is that association of α_{s1}-casein as a function of ionic strength was shown to be partially hydrophobic by light scattering [1] and by sedimentation equilibrium measurements [7]. It should follow that the association of α_{s1}-casein(136–196) may have a stronger temperature dependence than the parent. Heating to 60°C could induce further hydrophobic aggregation, and sharpening of spectral features for much of α_{s1}-casein(136–196) as a function of temperature may result from changes in the mobility and/or local secondary structure of the polypeptide chains accompanied by some 'side chain melting' rather than dissociation. Either hypothesis is plausible based upon current data.

3.3. *Estimation of secondary structure of the peptide*

Values of estimated bulk secondary structure for α_{s1}-casein(136–196) as calculated by far-UV CD and FTIR spectroscopies are given in Table 1. The global secondary structural results for native α_{s1}-casein obtained from Raman spectroscopy indicated 29–35% turns [19]. This result correlates well with proline residues being primarily involved in β-turns, as was discussed vis-à-vis the model shown in Fig. 2. The CD results for α_{s1}-casein(136–196) are also consistent with a putative structure in which all six proline residues are involved in turns. The far-UV (190–250 nm) CD measurements were made using a 43 μM (0.3 mg/ml) aqueous solution of α_{s1}-casein(136–196) at pH 6.75 in a low ionic strength buffer (2 mM potassium PIPES+4 mM KCl). At room temperature (27°C) results indicate the presence of approximately 58% β-sheet, 31% turns, 8% unordered and a statistically negligible amount of α-helix. Global secondary

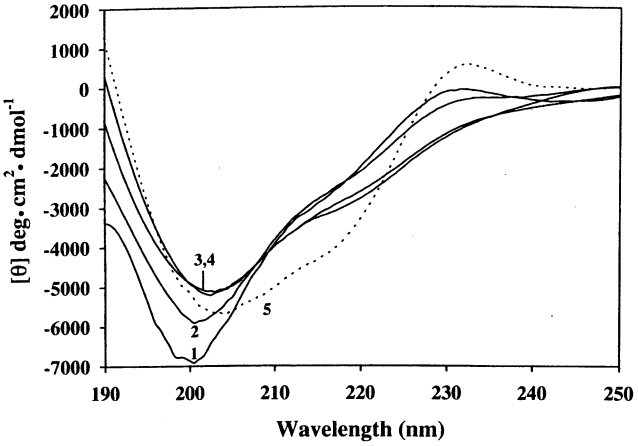

Fig. 6. Mean residue ellipticity [θ_r] as a function of wavelength for the far-UV CD of α_{s1}-casein(136–196) at 10°C, 27°C, 50°C, 70°C (solid lines 1 through 4, respectively); buffer conditions were 2 mM PIPES, 4 mM KCl at pH 6.75. Concentration of peptide was 0.260 mg/ml. The dashed line 5 represents 2 mM sodium phosphate, pH 2 at 27°C. The ellipticities are expressed in degree•cm^2/dmol mean residue weight.

structure was also estimated from the FTIR spectrum of a 1.5% w/w aqueous solution of α_{s1}-casein(136–196) at pH 6.5 and 25°C in 25 mM potassium PIPES buffer. The results of this analysis were as follows: 49% β-extended structures (extended, sheet, bent strand, 3_{10} helix), 22% turns, 23% unordered and a negligible amount of α-helix (5% of 60 = 3 residues). The calculated percentage of turns is lower for FTIR calculations than that obtained from far-UV CD, but still supports the assumption that most proline residues in α_{s1}-casein(136–196) may be involved in turn structures.

3.4. *Effects of temperature on CD spectra*

Fig. 6 shows the change in mean residue ellipticity as a function of wavelength in the far-UV for α_{s1}-casein(136–196) at four different temperatures. The calculated secondary structural features for each dichroic spectrum in Fig. 6 are given in Table 1. As the peptide is heated, there is an increase in the calculated amount of unordered (random coil) structure, indicating that there is thermal unfolding of local structures. The amount of random coil reaches a plateau at 50°C to 70°C, which compares well with the improved NMR spectral resolution observed at 50°C to 70°C in Fig. 5. While the increase in unordered structure with temperature occurs with a proportional decrease in sheet structure, the amount of calculated β-turn remains statistically invariant at about 30%. A similar conservation of turns at high temperature was previously observed for the hydrophobic protein β-casein (A^1 variant) by Graham et al. [28]. Conformational analysis by CD using the same Provencher and Glöckner data base [25] as used in this study, showed β-turn structures in intact β-casein to be statistically invariant for spectra obtained at 2°C, 25°C and 60°C.

In the energy-minimized, predicted structure of whole α_{s1}-casein (Fig. 2), both the Pro-147 and Pro-168 based β-turns are bordered by two regions of extended β-sheet. These sheet/turn/sheet motifs make up what are informally called the Pro-147 'arm' (residues 130 to 160) and the Pro-168 'arm' (residues 160 to 177). In the crystal structure for the insulin [29], each monomer contains a similar pair of extended β-strands at the monomer–mono-

Table 1
Comparison of secondary structural estimates for the peptide α_{s1}-casein(136–196) by three methods

Method	Temperature	% β-Sheet	% Turns	% Unspecified	% α-Helix
FTIR[a]	25°C	49 ± 3[b]	22 ± 1	23 ± 1	5 ± 2
CD[c]	27°C	58 ± 1	31 ± 1	8 ± 1	3 ± 1
	10°C	64 ± 2	28 ± 1	5 ± 2	3 ± 1
	50°C	49 ± 2	28 ± 1	18 ± 1	5 ± 1
	70°C	49 ± 2	29 ± 1	18 ± 1	3 ± 1
Sequence-based predictions and molecular modeling	In vacuo	57	28[d]	15	0

ent upon local secondary structure which stems from its primary amino acid sequence. Proton NMR results, in agreement with previous ultracentrifuge data [7], indicate that the peptide is aggregated even at very low concentrations (7 μM), and that aromatic spectral features may be used as an indicator of hydrophobic association at higher peptide concentrations. Temperature studies from both ^{1}H NMR and near- and far-UV CD indicate the thermal unfolding of local structures. There is a loss in the bulk quantity of extended structure (based on CD analysis) with increasing temperature, while β-turn structures are conserved at temperatures as high as 70°C. There is also a loss of side chain dichroism indicative of some 'side chain melting.' The retention of some conformational stability for backbone and side chains at 70°C, at pH 2 and in 6 M guanidine•HCl suggests a 'molten globule-like', heat stable, core structure for this peptide. It may be speculated that β-turns, directed by proline, represent a nucleation site for casein interactions as has been suggested for β-structures in staphylococcal nuclease [33] and most recently for β-lactoglobulin [34]. Structural elucidation of these important interaction sites in caseins will help to further understanding of the functional properties of these widely utilized food proteins as well as the core interactions necessary to form these unique calcium-transport complexes in vivo [23]. This information represents the first evidence for a 'molten globule-like' state in a casein. It may be used in the future to determine rational designs for genetic engineering modifications, which can ultimately direct protein structure and function.

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ent upon local secondary structure which stems from its primary amino acid sequence. Proton NMR results, in agreement with previous ultracentrifuge data [7], indicate that the peptide is aggregated even at very low concentrations (7 μM), and that aromatic spectral features may be used as an indicator of hydrophobic association at higher peptide concentrations. Temperature studies from both ^{1}H NMR and near- and far-UV CD indicate the thermal unfolding of local structures. There is a loss in the bulk quantity of extended structure (based on CD analysis) with increasing temperature, while β-turn structures are conserved at temperatures as high as 70°C. There is also a loss of side chain dichroism indicative of some 'side chain melting.' The retention of some conformational stability for backbone and side chains at 70°C, at pH 2 and in 6 M guanidine•HCl suggests a 'molten globule-like', heat stable, core structure for this peptide. It may be speculated that β-turns, directed by proline, represent a nucleation site for casein interactions as has been suggested for β-structures in staphylococcal nuclease [33] and most recently for β-lactoglobulin [34]. Structural elucidation of these important interaction sites in caseins will help to further understanding of the functional properties of these widely utilized food proteins as well as the core interactions necessary to form these unique calcium-transport complexes in vivo [23]. This information represents the first evidence for a 'molten globule-like' state in a casein. It may be used in the future to determine rational designs for genetic engineering modifications, which can ultimately direct protein structure and function.

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